Journal of the Egyptian Society of Haematology & Research, Vol. 14, No. 1, March: 9-15, 2018

Leukemic Cell/Microenvironment Cross Talk in Acute Myeloid Leukemia: Effect on CXCL12

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ABSTRACT

Background: Mesenchymal stromal cells (MSCs) secrete an array of signaling molecules; among these molecules is the chemokine CXCL12 whose fine-tuned concentration in bone marrow niches is important for maintaining stem cell characteristics. In acute myeloid leukemia, leukemic stem cells occupy the stem cell niche and react with the stromal cells at the expense of normal hematopoietic stem cells resulting in aberrant malignant niches. This crosstalk influences leukemogenic process and response to therapy.

Objectives: This work aims to demonstrate the effect of acute myeloid leukemia cells on mesenchymal stromal cells' production of CXCL12.

Subjects and Methods: MSCs were cultured from 20 AML bone marrow aspirates and 10 non neoplastic bone marrows as a control group. CXCL12 gene expression in cultured mesenchymal cell was evaluated using real time PCR and determination of the level of CXCL12 protein in BM plasma was performed using ELISA.

Results: CXCL12 gene expression was elevated in MSCs in AML cases in comparison to the control group; in addition, the level of CXCL12 in BM plasma was significantly higher in AML cases in comparison to the control group.

Conclusion: These findings suggest that MSCs is an active component in AML microenvironment through increasing the expression and levels of CXCL12 chemokine, which plays an important role in leukomogenesis.

Key Words: *Acute myeloid leukemia – Bone marrow microenvironment – CXCL12 – Mesenchymal stromal cells.*

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by the accumulation of clonal undifferentiated leukemic cells in the bone marrow (BM) and/or peripheral blood. Leukemic cells exhibit uncontrolled proliferation, blockade of differentiation capabilities, and reduced apoptotic ability. Despite initial responsiveness to standard chemotherapy, the prognosis of AML patients remains poor, considering the high relapse rates **[1-4]**.

Bone marrow niches, defined as the cellular and molecular micro-environmental components that regulate stem cell functions, control the balance between quiescence, self-renewal, and differentiation both in normal and malignant conditions. In acute myeloid leukemia, crosstalk between stromal cells and the leukemic stem cells (LSCs) within the bone marrow niches affects the leukemogenesis initiation and progression and influences the prognosis. This crosstalk provides a suitable environment through which LSCs acquire a drug-resistant phenotype and thus can evade the apoptotic signaling induced via chemotherapeutic drugs resulting in drug resistance or relapse status **[1,5-9]**. Several critical signaling pathways are involved in these interactions, among them is the CXCL12-CXCR4 signaling pathway **[10-12]**.

Stromal cell-derived factor-1 (SDF-1/CXCL12) is a CXC chemokine, which maintains the quiescent hematopoietic stem cell (HSC) pool via CXCL12-CXCR4 signaling pathway in bone marrow niches **[12,13]**. It is continuously secreted through marrow stromal cells. The vascular bone marrow niche includes heterogeneous stromal cells that are characterized by high CXCL12 expression, among these cells: CXCL12-abundant reticular (CAR) cells **[8,12]**, leptin receptor+ stromal cells and nestin-GFP+ stromal cells **[8,14]**. CAR cells, as mesenchymal progenitors that can differentiate into both

adipogenic and osteogenic cells in vitro, are the major source of CXCL12 in the bone marrow. Loss of CAR cells is associated with marked reduction in CXCL12 levels in the bone marrow **[11,15]**. CAR cells are in direct contact with HSCs and are important components of HSCs niches through which HSCs maintain their self-renewal capabilities **[12,15,16]**.

Association of CXCL12/CXCR4 with AML pathogenesis was indicated in several studies in which levels of CXCR4 expression on CD34+ cells were found as a negative prognostic indicator of the overall survival and disease-free survival **[17,18]**. The interactions between the LSCs and the bone marrow stromal cytokines/ chemokines and adhesion molecules have been claimed to be the cause of drug resistance and disease relapse in AML. Several studies reported that stromal elements protect AML from chemotherapy-induced apoptosis; CXCL12-CXCR4 pathway has been recognized as a critical mediator of this protection **[19-21]**. Moreover, CXCL12, through binding and activation of its receptor CXCR4 on leukemic cells, facilitates leukemia cell trafficking and homing in the bone marrow, and maintains leukemic cells in close proximity to the stromal microenvironment that continuously sends anti-apoptotic and pro-survival signals such as activation of the PI3K (phosphoinositide 3-kinase)/AKT and MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) axes **[22-24]**.

A better understanding of the interactions between LSCs and bone marrow stromal cells is required. Furthermore, focusing on stromal cells gene expression patterns that contribute to LSCs maintenance and quiescence may provide new therapeutic modalities to overcome drug resistance of current chemotherapeutic agents. This could be done through targeting these important interactions and disturbing the protective effect of the stromal cells on the LSCs **[25-27]**.

The aim of this work is to demonstrate the effect of acute myeloid leukemia cells on mesenchymal stromal cells production of CXCL12 through the evaluation CXCL12 gene expression in cultured mesenchymal cell using real-time PCR and determination of the level of CXCL12 protein in BM plasma using ELISA.

SUBJECTS AND METHODS

Subjects:

Twenty de novo AML cases were included in this study. Patients were diagnosed and selected among cases referred to Al-Kasr Al-Ainy Hospital, Faculty of Medicine, Cairo University. Samples were taken at time of initial diagnosis, before induction therapy. Diagnosis of AML was based on standard morphology, cytochemistry and immuno-phenotyping of leukemic blast cells. Patients' clinical and laboratory information were reviewed from their medical records. Ten control cases were included in this study; they were patients undergoing bone marrow aspiration for other indications than malignancy e.g. before splenectomy for hypersplenism or immune thrombocytopenia. An informed written consent was obtained from each patient or parents/guardians before enrollment. The work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in human patients. The ethical committee of Clinical and Chemical Pathology department, Faculty of Medicine, Cairo University approved the study. Patients' characteristics are summarized in Table (1).

Table (1): Clinical and laboratory characteristics of the studied groups.

Parameter	AML group N=20	Control group N=10
Age (years):		
• Median (range)	39.5 (10-62)	31.5 (20-56)
Gender:		
• Male: N (%)	10 (50%)	3 (30%)
• Female: N (%)	10 (50%)	7 (70%)
TLC (10^9/L):		
• Median (range)	70 (34 -163)	6 (4-8.5)
Blast percentage in bone marrow:		
• Median (range)	74.5 (34-85)	–
FAB classification: N (%)		
• M2	2 (10%)	
• M3	6 (30%)	
• M4	7 (35%)	
• M5	4 (20%)	
• M6	1 (5%)	

Methods:

Sample collection:

1-2 ml heparinized bone marrow samples were collected from the posterior iliac spine of

patients and controls; samples were processed within 24 hours of collection for mononuclear cell separation and cell culture. One ml citrated bone marrow sample was withdrawn for CXCL12 detection by ELISA.

Separation of human MNCs from bone marrow:

Heparinized marrow samples were diluted with double volume of RPMI with Lglutamine (Lonza, Cologne, Germany). Mononuclear cells were separated by Ficoll hypaque (Biowest, France). Cells were re-suspended in 2ml complete culture medium (CCM); Dulbecco's modified Eagle medium (DMEM) with lg/l glucose (Lonza, Cologne, Germany), 10% fetal bovine serum (FBS) (Lonza, Cologne, Germany), 1% L-Glutamine (Biowest, France) and 2% antibiotic-antimycotic (penicillin, streptomycin and amphotericin B) (Gibco, Eggenstein, Germany). Then the cells were counted **[28]**.

Culture and expansion of human MSCs:

The cells were plated in 25cm^2 tissue culture flask containing 5ml CCM with a density of 1.2X10^6 cells/cm^2. The flasks were incubated at 37ºC in 5% humidified CO2. After 48 hours, non-adherent cells were discarded. Five ml of fresh CCM was added to the flask and the flasks returned to the incubator. The cells inside the flask were examined every other day under inverted microscope. Every third day the medium was discarded, the cells rinsed with RPMI and fed with a fresh 5ml CCM until 80% confluency was reached. MSCs were characterized and identified by their plastic adherence and by their spindle fibroblast like morphology under the inverted microscope **[29]**.

MSC colonies were washed once with RPMI and harvested using Trypsin (Thermo Fisher Scientific Inc). Cells were cultured for three additional passages, tested for viability using trypan blue and cryopreserved using DMSO (Thermo Fisher Scientific Inc) and placed in cryo tubes at –80ºC for future experiments **[30]**.

Detection of CXCL12 gene by real time RT-PCR:

Thawing of cryopreserved cells:

Cells were rapidly thawed in water bath at 37°C over 2min. and transferred to a 50ml sterile falcon tube. Thawing media, HBSS1X (18ml, Thermo Fisher Scientific Inc) and FBS (2ml), was added after filtration (10x amount of cryopreserved cell suspension) without delay by drop wise addition **[21]**. Cells were centrifuged at 1500rpm for 5min at 4ºC, supernatant was removed and cells were re-suspended in remaining solution. Ten ml iced cold medium were added and viability determined. Cells were spun down at 1200rpm for 5min at 4ºC and the cell pellet used for RNA extraction.

CXCL12 gene expression by real time RT-PCR:

RNA extraction from the cultured MSC was done using QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany). cDNA was formed using reverse transcription kit (Applied Biosystem, USA). CXCL12 was detected through using Taqman gene expression assay for CXCL12 and β-actin as a house keeping gene (Applied Biosystem, USA) using Real time 7300 (Applied Biosystem, USA).

Data were presented as the comparative threshold cycle (Ct); the numerical value of the CT is inversely related to the gene expression in the reaction (i.e., the lower the CT, the greater the expression of the gene). Each sample was normalized with the housekeeping gene (β-actin) using the formula Δ Ct = Ct of CXCL12-Ct of β-actin. Ct was used to determine the gene expression relative to a normal control (calibrator). Transcription level of CXCL12 gene was represented by the following formula 2 to the power of –ΔΔCt as fold change from the control group Therefore; a 2-ΔΔCT value of more than 1 is considered as high gene expression in comparison to the control and a value of less than 1 is considered low expression of the gene **[31]**.

CXCL12 protein level in BM plasma using ELISA technique:

CXCL12 protein levels were detected in bone marrow plasma using ELISA technique (Wuhan USCN Business Co., Ltd, USA), Reading of samples was done using standard curve. Detection range of the kit was 0.156-10ng/mL.

Statistical analysis:

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data was expressed as median and range. Qualitative data was expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables. For not normally distributed quanti-

tative data, comparison between two groups was done using Mann-Whitney test (non-parametric *t*-test). Spearman-rho method was used to test correlation between numerical variables. All tests were two-tailed. A *p*-value <0.05 was considered significant.

RESULTS

CXCL12 gene expression in cultured MSCs in AML patients:

The expression of CXCL12 in cultured MSCs of AML cases was elevated in comparison to the control group (median fold change: 71.35, IQ range: 8.83-437.24). The highest expression levels of CXCL12 were detected among M4 patients, followed by M3 then the M5 subtype (Table 2).

There was no statistically significant correlation between gene expression levels of CXCL12 in MSCs and age of the patients (*r*= 0.052, *p*-value=0.844), TLC (*r*=–0.196, *p*-value =0.450) nor the blast percentage in the BM of AML group (*r*=–0.149, *p*-value=0.569).

There was no significant correlation between expression of CXCL12 gene in MSCs in BM using RT-PCR and the level of CXCL12 protein in BM plasma (*r*=0.081, *p*-value 0.756).

CXCL12 level in BM plasma:

The level of CXCL12 in BM plasma was significantly higher in AML de novo cases than in the control group (*p*-value <0.001). The level of CXCL12 in BM plasma of AML cases ranged from 0.6 to 10.8ng/ml (median: 6.9ng/ml). While for the control group, it ranged between 0.1 and 1.2ng/ml (median: 0.7ng/ml). As regards the level of plasma CXCL12 in different AML FAB subtypes: The median level of CXCL12 was highest among M3 cases followed by M4 cases. The least value was observed in the M5 subtype (Table 2).

No statistically significant correlations were found between the level of CXCL12 and either the age of the AML patients (*r*=0.149, *p*-value =0.532), TLC (*r*=0.19, *p*-value=0.939), or the blast counts in the bone marrow (*r*=0.092, *p*-value=0.699).

No statistically significant difference in the level of CXCL12 between males and females in AML group (*p*-value=0.393) as in males the levels ranged between 0.6 and 10.8ng/ml with median value of 5.9ng/ml, while in females levels ranged between 0.8 and 10.8ng/ml with median value of 8.9ng/ml.

Table (2): CXCL12 gene expression level in cultured MSCs and CXCL12 BM plasma level among AML subtypes.

Parameter	CXCL12 gene expression ($2^{-\Delta\Delta Ct}$) in cultured MSCs*	CXCL12 in BM plasma ng/ml*
FAB subgroup:		
M3 (No=6)	180 (36-507)	10.6 (0.8-10.8)
M4 (No=7)	389 (21-612)	5.3 (0.6-10.8)
M5 (No=4)	99 (50-184)	3.1 (0.9-10.8)

* Median (range).

DISCUSSION

During AML pathogenesis, leukemic cells progressively hijack and alter the normal hematopoietic niche where normal HSCs settle **[32,33]**. Growing evidence suggests that leukemia cells induce dramatic modulations in hematopoietic and non-hematopoietic cells within the niche at the genetic levels. These changes contribute to the appearance of leukemic niche that supports the leukemogenesis and blocks the normal hematopoiesis. However, the detailed underlying mechanisms remain largely unknown **[33-35]**.

Many researchers supported the idea of interactions between leukemic cells and bone marrow microenvironment so it is rational to give the assumption that MSCs in acute myeloid leukemia exhibit molecular changes due to their exposure to the leukemic cells **[36-37]**. These changes, in turn, may represent an essential component of disease relapse and drug resistance characterizing AML disease **[38]**.

In this study, we demonstrated an increased expression of CXCL12 mRNA in the MSCs in AML patients in comparison to the control group. This is in accordance with Civini et al., who proved that leukemic cells affect the gene-profiling pattern in stromal mesenchymal cells that lead to the expression of multiple cytokines and chemokines including CXC12. The study also noted that this effect on mesenchymal cells differs according to the degree of stemness of the leukemic cells resulting in the heterogeneity of clinical presentation among leukemia sub-

types **[39]**. Moreover, Kim et al., demonstrated that leukemia stem cells from de novo AML patients can induce extensive alterations in the mesenchymal niche, resulting in an altered expression of crosstalk molecules, including CXCL12 **[40]**.

In another study, Lopes et al., determined cytokine expression profile of MSCs from patients with myelodysplastic syndrome (MDS), AML with myelodysplasia-related changes (MRC) and de novo AML, in comparison to healthy control. They found that MSCs in AML-MRC showed a significant increase in IL6 expression, whereas de novo AML MSCs presented a significant increase in the expression levels of different cytokines including VEGFA, CXCL12, RPGE2, IL1β, IL6, and IL32. They suggested that the difference in pathogenesis of AML-MRC and de novo AML may extend into MSCs within the leukemic niche **[41]**.

These results indicate that crosstalk between the leukemia cells and MSCs is a dynamic process. MSCs affect leukemia cells and leukemia cells change MSCs genetic profile to be in favor of leukemogenesis.

In this study, CXCL12 protein level in the BM plasma of AML patients was significantly elevated at initial diagnosis in comparison to the control group. However, we did not find a significant correlation between the level of CXCL12 protein and the blasts percent in the bone marrow or with the peripheral blood total leucocytic count. This is in accordance with Alinkovich et al who found that the concentrations of CXCL12 in the peripheral blood and bone marrow plasma samples from AML patients were significantly elevated in comparison to the normal group. Furthermore, they found that CXCL12 was able to enhance in vitro survival of AML cells **[42]**. Also, Wen et al., studied the level of plasma CXCL12 in 48 children with acute leukemia and 20 with non-hematologic malignancies as a control group and showed that the level of CXCL12 in the peripheral plasma of acute leukemia group was significantly higher than that of the control group **[43]**.

In our study, although CXCL12 expression in MSCs and the level of CXCL12 in bone marrow plasma of AML cases were higher than the levels in the control group, they are not correlated to each other statistically. This indicated that MSCs contribute to CXCL12 secretion but they are not the sole source of CXCL12 in bone marrow of AML cases. It has been shown that vascular endothelial cells, stromal fibroblasts, and osteoblasts are contributor sources of CXCL12 **[10,44]**.

The highest expression of CXCL12 gene was detected among our M4 AML cases followed by M3 AML cases, while the level of CXCL12 in BM plasma was highest in promyelocytic AML (FAB: M3) followed by M4 and M5 AML. Mohle et al., observed differential expression of CXCR4, CXCL12 receptor, in different FAB subtypes being higher in AML (M3) and myelomonocytic AML (M4/5), while its expression is low in AML (M0, M1, and M2) **[45]**. Concentrations of CXCL12 protein in the bone marrow plasma in AML patients could be sufficient to provide survival and anti-apoptotic signals to AML cells. Few data are available regarding the role of CXCL12 in different AML subtypes. Heterogeneity of the alteration in mesenchymal stromal cell function in different subtypes of AML at initial diagnosis might be the contributing factor to their heterogeneous presentation and the further heterogeneous post-treatment outcome, and hence may serve as a potential prognostic factor **[40]**. In addition, correlation of tumor cell aggressive phenotype and metastasis with the levels of CXCL12 expression was documented in different studies, among different types of tumors **[46-48]**.

Further studies on larger groups of different AML subtypes are recommended to further elucidate the differential role of CXCL12 in different FAB subtypes pathogenesis.

In conclusion, our study demonstrated that bone marrow mesenchymal stromal cells react to myeloid leukemic cells by increasing the expression of CXCL12 and CXCL12 protein level. This alteration of CXCL12 may contribute to the creation of a BM niche more favorable to host leukemia stem cells.

Conflict of interest: The authors declare that they have no conflict of interest.

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